International Scholars Journals

International Journal of Medicinal Plants Research ISSN 2169-303X Vol. 8 (6), pp. 001-007, June, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana*

Mousumi Debnath*

Plant Biotechnology Laboratory, Department of Biotechnology, JECRC Foundation, Mahatma Gandhi Institute of Applied Sciences, Shri Ram Ki Nangal, Via Vatika, Tonk road, Jaipur, India 303905. E-mail: mousumi.debnath@gmail.com.

Accepted 14 January, 2019

A procedure has been outlined for plant regeneration and antimicrobial screening of a medicinal herb, *Stevia rebaudiana* Bertoni, through *in vitro* culture of nodal segments with axillary buds. Murashige and Skoog (MS) medium supplemented with 2.0 mg/L N^6-benzyl amino purine and 1.13 mg/L indole-3-acetic acid in combination were found to be most effective in inducing bud break and growth, and in initiating multiple shoot proliferation at the rate of 39 microshoots per nodal explant after 30 d of culture. By repeated subculturing a high-frequency multiplication rate was established for production of elite lines of *Stevia rebaudiana*. Elongated shoots were transferred to rooting medium. MS medium supplemented with 2.0 mg/L indole-3-butyric acid was found to be best for rooting. *In vitro* and *in vivo* grown leaf extracts in different solvent system were screened for potential antimicrobial activity against medically important bacterial and fungal strains by agar well diffusion method. The chloroform and methanol extract exhibited a concentration dependent antibacterial and antifungal inhibition. Both *in vitro* and *in vivo* dried leaf extract showed similar antimicrobial activity. Therefore, commercial manufacture of active constituents from these improved elite lines would be useful and profitable.

Key words: Regeneration, antimicrobial, *Stevia rebaudiana*, medicinal plant.

INTRODUCTION

In ancient Indian traditional Ayurvedic system of medi-ine, *Stevia rebaudiana has* a long history of use by tribal people*. Stevia rebaudiana* Bertoni, belonging to the family Asteraceae, is a perennial and endemic, medicinal shrub (Sivaram and Mukundan, 2003). Medicinal plants constitute one of the most important groups of wild plants in terms of their contribution to the economy and well-being of farmers.

Stevia is a natural non-calorie sweetener (Chalapathi and Thimmegowda 1997) and is indigenous to the northern regions of South America. The crop was first established in Japan in 1968. By mid- 1970s, standar-dized extract and pure stevioside was utilized comer-cially in Japan for sweetening and flavoring foods and be-verage as a substitute for several synthetic sweete-ners. The total market value of *Stevia* sweetener in Japan is established to be around 2 - 3 billion yen/year. It is being cultivated in continental China, Taiwan, Thailand, Korea, Brazil, and Malaysia. Besides the above-mentioned coun-tries, *Stevia* today is also grown in Israel, Ukraine, UK, Philippines, Canada, Hawaii, California, and all over South America. The leaves of Stevia contain diterpene glucosides viz. stevioside and rebaudioside with a sweet taste but which are not metabolized. The biggest part of the sweet glycosides consists of the stevioside molecule (Brandle et al, 1998). The sweetener, stevioside (Nepo-vim and Vanek, 1998) extracted from the plants is 300 times sweeter than sugar. The fresh leaves have a nice liquorice taste. It is recommended for diabetes and has been extensively tested on animals and has been used by humans with no side effects (Megaji et al., 2005). For anyone who suffers from diabetes, hypoglycemia, high blood pressure, obesity and chronic yeast infections, *Stevia* is the ideal sweetener.

The seeds of *Stevia* show a very low germination per-centtage. Propagation by seeds does not allow the pro-duction of homogeneous populations, resulting in great variability in important features like sweetening levels and composition (Nakamura and Tamura, 1985). Vegetative

ABBREVIATIONS: Murashige and Skoog's medium (MS),Kinetin (Kn), 6-benzylamino purine (BAP), indole -3- butyric acid (IBA), indole acetic acid (IAA) naphthalene acetic acid (NAA).

propagation too is limited by the lower number of individuals that can be obtained simultaneously from a single plant (Sakaguchi and Kan, 1982). Due to the above-mentioned difficulties, tissue culture is the only alternative for rapid mass propagation of *Stevia* plants. Plant tissue culture technology may help to conserve rare and endangered medicine plants. Many important medicinal herbs have been successfully propagated *in vitro*, either by organogenesis (Debnath et al., 2006) or by somatic embryogenesis.

Tamura et al. (1984) established clonal propagation of *S. rebaudiana* by culturing stem tips with an increasing demand for stevioside in the food industries. Clonal propagation of *S. rebaudiana* has been established by culturing stem tips with a few leaf primodia on agar me-dium supplemented with a high concentration (10 mg/l) of kinetin. Ferreira and Handro (1988) described a method for production maintenance and plant regeneration from cell suspension cultures of *S. rebaudiana* (Bert.) Bertoni. Bespalhok et al. (1993) establish somatic embryogenesis from leaf explants of *S. rebaudiana.* Bespalhok and Hattori, (1997) obtain embryogenic callus formation and histological studies from *S. rebaudiana* (Bert.) Bertoni floret explants. Patil et al. (1996) and Sivaram and Mukundan (2003) reported in *vitro* culture studies on *S. rebaudiana.*

Stevia is thought to inhibit the growth of certain bacteria and other infectious organisms. Some people even claim that using *Stevia* helps to prevent the onset of colds and flu. *Stevia* has the ability to inhibit the growth of certain bacteria, helps to explain its traditional use in treating wounds, sores and gum disease. It may also explain while the herb is advocated for anyone who is susceptible to yeast infections or reoccurring streptococcal infections, two conditions that seem to be aggravated by white sugar consumption. The biological activity for compound *Stevia* has been studied by Tomita et al. (1997). They have studied bactericidal activity of a fermented hot-water extract from *S. rebaudiana* Bertoni towards enterohemorrhagic *Escherichia coli* and other food-borne pathogenic bacteria. Other microorganism like *Salmonella typhimurium, Bacillus subtilis, and Staphylococcus aureus* has been found to be inhibited by fermented leaf extract. Similar antimicrobial studies of leaf extract of *Ocimum gratissimum* on selected diarrhea causing bacteria in South-Western Nigeria (Adebolu and Oladimeji , 2005) has also been studied. The medicinal properties are attributed to the primary and secondary metabolites synthesized by the plants (Faizi et al., 2003). Antibacterial activity of various plant extracts has been studied by many workers (Brantner and Grein, 1994; Brantner et al., 1996; Erdogrul, 2002). The effects of plant extracts on bacteria have been studied by a very large number of researches in different parts of the world. Much work has been done on ethanolic extracts plants in India (Pareek et al., 2005).

Interest in a larger number of traditional natural products has that aqueous and ethanolic extracts from plants used in allopathic medicine are potential sources of antitumoral agents (Brantner and Grein, 1994). The selection of crude plant extracts for screening programs has the potential of being more successful in initial steps than the screening of pure compounds isolated from natural products (Sah et al., 2005). The objectives of the present study include *in vitro* organogenesis of *S. rebaudiana* and comparison of antimicrobial activity of dried leaf extract (*in vitro* and *in vivo)* in different solvent system.

MATERIAL AND METHODS

In vitro studies

S. rebaudiana plants were a kind gift from a nursery of Bhopal. It was identified and characterised and *in vitro* work was initiated. Shoot apex, nodal, and leaf explants ranging in size from 1 to 1.2 cm were collected from a young growing plant. The mother plants were maintained in the greenhouse under a temperature of 28 $\pm$ 5^0C, at a relative humidity of 50% and partial shade conditions. They were watered twice a day. After excision, they were rinsed in running tap water for 20 min and immersed in Tween 20 solution for 10 min. After three washes with double-distilled water, further sterilization was carried out in the laminar airflow chamber using 0. 1% (w/v) $HgC1_2$ for 5 min. The explants were then rinsed three times with sterile water. Inoculation of *Stevia* explants was done on MS medium (Murashige and Skoog, 1962), supplemented with cytokinins and auxins used singly and in combination. The pH of the nutrient medium was adjusted to 5.8 and 0.8% agar (Himedia, Mumbai) was added prior to autoclaving. The cultures were maintained at a temperature of 25^0C and 16 h photoperiod. Various concentrations of growth regulators, viz. 6-benzyladenine purine (BAP), kinetin (Kn), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) were tried for shoot proliferation and callus induction.

Preparation of dried leaf extracts of *S. rebaudiana* and antibiotic and antifungal solutions

The dried leaves of *in vitro* and *in vivo* raised *S. rebaudiana* were subjected to different extraction methods, using various solvents viz. distilled water, chloroform, methyl alcohol. Leaves (2.0 g) were collected, dried and subjected to cold extraction at 4^0C for 24 h. The paste was filtered using cheesecloth and was centrifuged (10,000 rpm, 10 min, 25^0C). The supernatant obtained after centrifugation was concentrated for antimicrobial assay. The various dilutions for *Stevia* leaf extract in different solvent were also prepared. Stock solutions of wide spectrum antibiotics like tetracycline, ampicillin, streptomycin and nystatin were prepared as 10.0 mg/ml (w/v) concentration in sterile distilled water and filter sterilized by using syringe filters. The concentration of 1 μL/well was used for the antibacterial assay in each well.

Antimicrobial activity

Four pathogenic bacterial and six fungal strains were tested against different extracts of S. rebaudiana. Certified strains of bacterial viz. *E. coli* (MTCC 41), *B. subtilis* (MTCC 441), *S. mutans* (MTCC 497), *S. aureus* (MTCC 737) were obtained from IMTech, Chandigarh. Certified strains of fungi viz. *Sclerotonia minor, Curvularia lunata, Alternaria alternate, Aspergillus niger, Microsporium gypsium, Rhizopus* were obtained from Department of Microbiology, R.D. University, Jabalpur, India. Various media viz. nutrient agar (*E. coli*, *B. subtilis*, *S. aureus)*, Potato Dextrose Agar (*Sclerotonia minor*,

Table 1. Effect of various concentrations of hormones for shoot proliferation in *S. rebaudiana*.

Plant growth hormone (mg/L)			% sprouted	Mean number of shoots	Mean length of shoots (cm)	Frequency of callogenesis
BAP	**Kn**	**IAA**				
0.1	-	-	10	1.5	0.2	-
0.5	-	-	20	4.2	1.2	-
1	-	-	15	4.5	0	+
1.5	-	-	20	5	1.7	-
5	-	-	0	0	0	-
-	0.5	-	0	0	0	-
-	2.0	-	18	1.5	0.5	-
-	10	-	0	4	1.2	+
0.1	-	0.1	12	1.5	1.5	+
0.5	-	0.2	13	8.5	2.1	++
1.0	-	0.3	30	9	2.0	+
1.5	--	0.4	40	20	2.0	+
2.15	-	3.68	45	25	3.5	-
2.0	-	1.13	90	39	3.8	-

Curvularia lunata,*Alternaria alternate*, *Aspergillus niger*, *Microsporium gypsium*, *Rhizopus niger*) and , Brain Heart Infusion Agar (*S. mutans*) were used for maintenance of the cultures. Stock cultures were maintained on nutrient agar slants. Subculturing was done once a month to maintain their viability and to check for their purity. All the experiments were done when the microorganisms were in the logarithmic phase. Overnight cultures were prepared by transferring a loop full of stock cultures to tube having nutrient agar and incubating at 37^0C for 18 - 24 h. These cultures were then used as inoculum for culturing pathogens on Petri dishes for the antimicrobial test. Antimicrobial activity against the test organism was performed using the agar diffusion method (Perez et al., 1990). For antimicrobial studies, the nutrient broth was inoculated with each of the pathogenic strain and grown overnight at 37^0C. The optical density was measured at 610 nm and cultures were adjusted at same optical density for the equal growth of all pathogens. The micro-organisms (pathogens) were used to seed different nutrient agar plates; one organism per plate, wells were made on the plates with a sterile cork borer of 4 mm diameter to contain the different extracts and the plates were incubated at 37°C for 24 h. The zones of inhibition were measured at the end of the incubation period. The same procedure was repeated with all extracts and pathogens as well with commercial antibiotics. Microbial growth was determined by measuring the diameter of the zone of inhibition and the mean values are presented (Table 3, 4) and were compared with standard antibiotics like ampicillin (antibacterial) as well as nystatin (antifungal). The effects of the solvents (without extract) on the growth of micro-organisms were also recorded.

RESULT

Establishment of *in vitro* culture

Various explants viz. shoot apex, nodal, and leaf extract was used as explants. They were subjected to $HgCl_2$ treatment. The cultured explants showed 40 percent contamination free cultures when treated with 0.1% $HgCl_2$ for 5 min for surface sterilization. The stem tips without leaf primordia (less than 0.3 mm in length) failed to grow and turned brown. However, 60% of the stem tips with 2 to 4 leaf primordia developed multiple shoots. Nodal seg-ments with 2 - 4 leaf primordia resulted in multiple shoot proliferation.

In vitro propagation studies show that the plant hormones play an important role in the callogenesis and regeneration. The various combinations of the hormones used for regeneration of shoot tip are given in Table 1. Of the two cytokinins tried BAP proved to be better than Kn for shoot induction from shoot induction and regeneration from shoot apical meristem, nodal and leaf explants, while BAP could induce shoot regeneration in all the three explants kinetin could do so only in few shoot apex and nodal explants.

Various concentrations of hormones (auxins and cytokinins) were tried for initiation and proliferation of shoots. Multiple shoots were found to be developing from shoot apex and nodal explant when cultured on MS medium with 2.0 mg/L BAP and 1.13 mg/L IAA. However, initiation of multiple shoots in most of treatments was observed within two weeks of culture (Figure 1A) and resulted in around 39 microshoots in 4 weeks (Figure 1 B). The shoot proliferations from shoot apex were also observed in MS medium supplemented with 1.5 mg/L BAP and 0.4 mg/L IAA (Figure 1C). In this treatment 70% of the cultured explants produced shoots where number of shoots per culture was 3 – 4 and average length of shoots per culture was 3.5 cm when transferred to the MS medium supplemental with 2.15 mg/L BAP and 3.68 mg/L IAA. After 25 days of growth (Figure 1E, F), the elongated shoots were transferred to the rooting medium. From majority of the leaf explants, callogenesis was observed. Best callogenesis was observed in MS medium supplemented with 2.05 mg/L 2,4-D Callogenesis was induced

Table 2. Effect of IBA on root proliferation in elongated segments of *S. rebaudiana* (reading after 45 days).

S/no	Concentration of IBA (mg/L)	Type of root	% of roots sprouted	Mean length of roots (cm)	Mean number of roots	Callus intensity
1	0.5	-	0	0	0	-
2	1	-	0	0	0	-
3	1.5	Tap	20	1.5	2	++
4	2	Tap	96	12.5	8	++++
5	2.5	Tap	18	4.5	2	+
6	3	-	0	0	0	-

Table 3. Antimicrobial activity of dried leaf extract of *S. rebaudiana* in different solvent system (Extract concentration 5%, reading after 24 h of growth).

S/no.	Strain	Inhibition zone (mm)in different solvent system							
		Chloroform extract		Methanol extract		Aqueous extract		Standard antibiotic	
		in vivo	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*
1	*E. coli (41)*	4	5	7	4	0	0	17	8.5
2	*S. mutans (497)*	7	8	10	11	0	0	20	10
3	*B. subtilis(441)*	6	2	7	3	0	0	16	7
4	*S. aureus (737)*	11	9	12	9	0	0	15	7
5	*Aspergillus*	0	0	0	0	0	0	2	1
6	*Curvularia*	0	0	15	16	0	0	2	1
7	*S.minor(1125)*	2	1	5	6	0	0	2	11
8	*Rhizopus*	0	0	0	0	0	0	13	6
9	*Alternaria*	0	0	0	0	0	0	2	1
10	*Microsporium*	0	0	0	0	0	0	2	1

induced *in vitro* leaves of *Stevia rebaudiana* were cultured on MS medium containing 2,4-D and BAP Callus formation was observed in 2,4D (4 mg/l) + BAP (1.0 mg/l) after 15 days of culture. The majority of calluses result in 2 - 3 shoot buds (Figure 1D). However, in our study *in vitro* multiple shoot proliferation of leaves and generation of new elite plant was the main objective. Root formation was induced in the *in vitro* proliferated shoots by culturing them on the ½MS medium containing IBA (0.1 – 2 mg/l) and IAA (0.1 – 2 mg/l). Best response was observed in IBA (2.0 mg/l) supplement with ½ MS medium as in this almost 90% of the inoculated explants showed root initiation (Figure 1F). The number of roots per culture ranged from 2 to 8 (Table 2, Figure 1H). The roots were of 0.2 - 0.5 cm in thickness and showed a range of variable length from 1 – 5 cm. Usually the roots were variable and appeared in bunches of 3 long and 5 short roots (Figure 1G, H). There was no branching of roots. The plantlets were hardened, acclimatized and established in soil (Figure 1I).The *in vitro* leaves were collected, air dried and processed for antimicrobial study.

Antimicrobial activity

The crude leaf extract of *S. rebaudiana* in different solvent system proved to be very effective antimicrobial agent. The methanolic extract was the most effective against all the bacteria. The activity was similar to antibiotic tested. Among the bacterial species maximum inhibition was found in *S. aureus*, followed *Streptococcus mutans*, *B. subtilis* and *E. coli* (Table 3). The crude leaf chloroform extract of *S. rebaudiana* leaves was also found to be effective than aqueous extract for all the bacteria tested. Maximum inhibition on comparative scale was found in *S. aureus* , *S. mutans* , *B. subtilis* followed by *E. coli* on the basis of the zone of inhibition (MIC).The activity of the extract was found to be comparable with 1% concentration of antimicrobial used (streptomycin, nystatin). The aqueous extract was found not effective to all the microorganisms tested. When the growth of the micro-organisms against the solvents (without leaf extract of *S. rebaudiana*) was evaluated, no marked inhibition of growth was observed. On various dilutions of the crude leaf extracts similar results confirmed. It was also observed that on dilutions the inhibition in some cases was more (Table 4). The aqueous extract was found to be non-effective for all fungi and bacteria. The methanolic extract was most were effective against all the fungi. The activity was similar to antifungal agent tested. Maximum zone of inhibition was shown by *Sclerotinia minor*. The

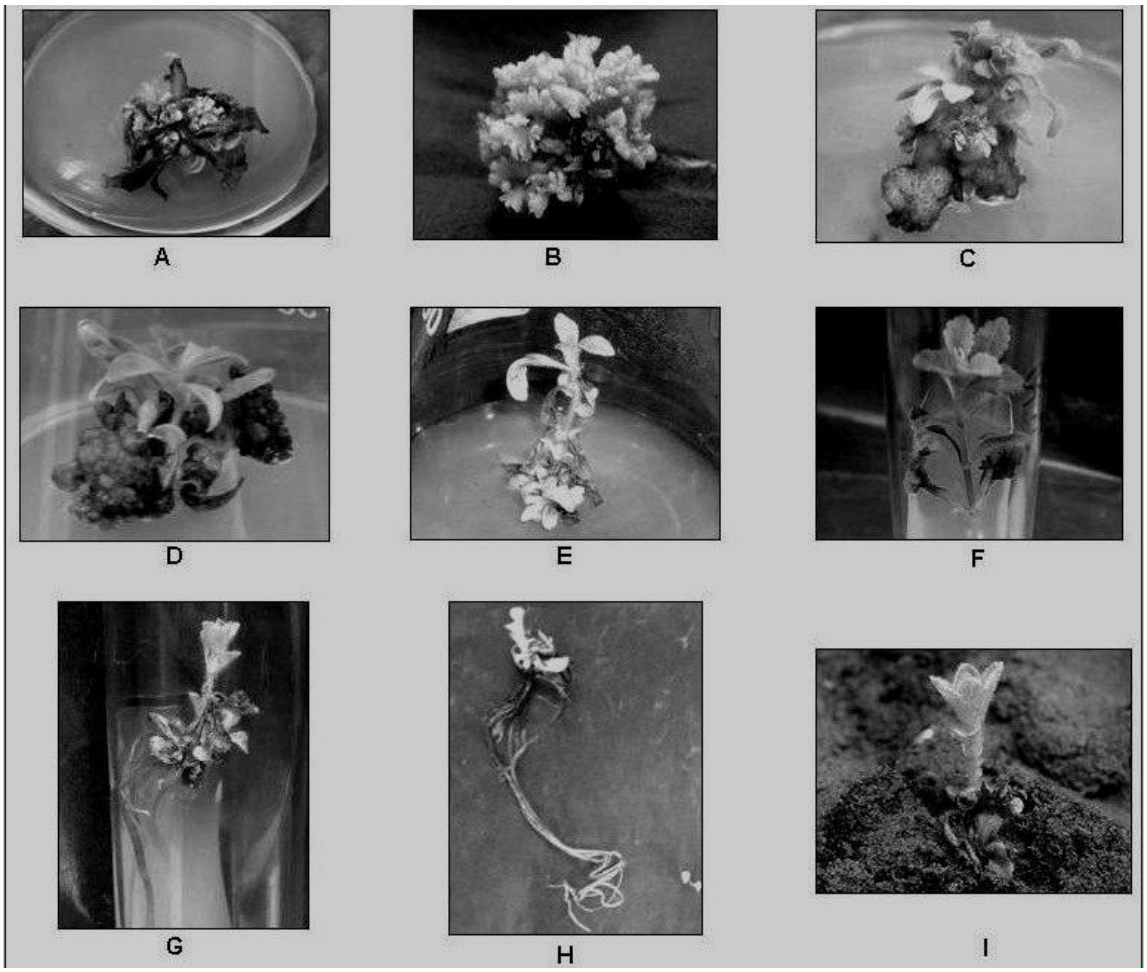

Figure 1. Clonal propagation of S. rebaudiana A. Nodal segments with axillary bud showing multiple shoot proliferation in MS medium supplemented with 2.0 mg/L BAP and 1.13 mg/L IAA (2weeks);B. Growth on the same medium after 4 weeks; C. Shoot proliferation from shoot apex in MS medium supplemented with 1.5 mg/L BAP and 0.4 mg/L IAA; D. Callus formation was in 2, 4-D (4 mg/l) + BAP (1.0 mg/l) ;E. The regenerated shoots were subcultured on to the MS medium supplemented with 2.15 mg/L BAP and 3.68 mg/L IAA and elongation observed (25 days);F. Initiation roots in MS supplemented with 2 mg/L IBA (25 days);G and H. Multiple rooting from the (45 days);I. Establishment in soil.

chloroform extract on dilution showed mild inhibition against the both bacterial and fungal growth. Maximum inhibition zone was shown by *S. aureus S. mutans*, *B. subtilis* followed by *E. coli* on the basis of the zone of inhibition. Maximum inhibition zone for fungal species was found in *Curvularia lunata* followed by *Sclerotinia minor*. *Curvularia lunata* showed inhibition against Stevia extract only in methanolic solvent and not in aqueous or chloroform solvent system. This is a clear indication that the solvent system plays an important role in the solubility of the antimicrobial substance and also affects the antimicrobial activity. *C. lunata* did not show any antimicrobial effect on dilution of the extract.

When similar studies were done to assay the antifungal growth against the various solvent extract of *Stevia* leaf, it was found that *Rhizopus and Microsporium gypsium* were most resistant against the crude as well as diluted extract. There was minimal inhibition in *Alternaria alternata* and *Aspergillus niger*. Maximum inhibition was observed in *Curvularia lunata* and *Sclerotinia minor* . The in *vivo* and *in vitro* regenerated leaf extract showed similar antimicrobial property .The minimum inhibitory concastration was found to be 0.1% in majority of the cases. In some cases the activity of the *in vitro* leaf extract was even more potent and effective as antimicrobial substance than *in vivo* grown leaf extract.

DISCUSSION

In this study experiments were conducted to standardize the explant source and culture media for multiple prolixferation of shoot and result in mass propagation of homogenous elite plantlets of *S. rebaudiana*. Ninety percent of the nodal explant (less than 1 cm.) with 6 to 8 leaf primor-

Table 4. Effect of different concentration of solvent concentration of *S. rebaudiana* extract on microbial growth (reading after 24 h of growth).

S/ no.	Solvent system	Conc. (%)	Zone of inhibition (mm)											
			E. coli (41)		*S. mutans* (497)		*B. subtilis* (441)		*S. aureus* (737)		*S. minor* (1125)		*Curvularia*	
			in vivo	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in viro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*
1	Methanol	1	4	2	10	18	6	5	14	19	5	3	0	0
2	extract	0.1	5	1	2	1	5	3	3	1	2	1	0	0
3		0.01	0	0	0	0	0	0	0	0	0	0	0	0
4		0.001	0	0	0	0	0	0	0	0	0	0	0	0
5		Standard antibiotic	12	9	15	13	10	8	2	2	2	2	2	2
6	chloroform	1	10	8	12	9	3	2	10	11	2	1	0	0
7	extract	0.1	5	3	9	6	2	1	5	6	0	0	0	0
8		0.01	2	0	0	0	0	0	2	0	0	0	0	0
9		0.001	0	0	0	0	0	0	0	0	0	0	0	0
10		Standard antibiotic	12	9	10	10	15	13	10	15	10	10	2	2

dia formed single shoots instead of multiple shoots. Tamura et al. (1984) also found out similar relationship between size of stem- tips and number of leaf primordia and came to the same conclusion. Patil et al. (1996) used 3 - 4 auxiliary bud as explant for maximum regeneration, whereas Sivaram and Mukundan (2003) used shoot apex, nodal and leaf explant ranging in size from 1 to 1.2 cm.

It seems as if the larger explants contain unknown substances that inhibits the formation of multiple shoots as in the expression of apical dominance callus formation was also observed in some of the experiments. These results suggest that the ability to form multiple shoots is dependent on the size of excised stem tip and / or the number of leaf primordia (Tamura, 1984).Similar studies on shoot proliferation has been performed by Patil (1996), Nepovin and Vanek (1998), Sikach (1998), Akita and Shigeoka (1994), Ferreira and Handro (1998) and Sivaram and Mukundan (2003). They also reported that plant hormone is necessary for shooting, elongation and rooting. In most of cases BAP was found to be essential for growth and was better than Kn for shoot induction for various explants. Combination of BAP and IAA has also been reported to be better for shooting and elongation. Callus formation has also been observed by Xie et al. (1998), Bespalhok and Hatori (1997), Ouyang (1996), Ferreira and Handro (1998) from leaf and floret (Flachsland et al., 1996) of *S. rebaudiadia*. Probably the callus observed most of our cases may be embryogenic in nature. Though on multiple reproductions of same result as well as histological studies could only confirm it. Sivaram and Mukundan (2003) also found out similar response when the elongated shoots were transferred to half strength MS medium, supplemented with IBA. Earlier Ferreira and Handro (1998) succeeded in achieving rooting in medium containing BAP and IBA.

The medicinal properties are attributed to the primary and secondary metabolites synthesized by the plants (Faizi et al., 2003). In our studies we found that all the 4 bacteria were inhibited by the *S. rebaudiana* extract in the various solvent although only a few fungi showed inhibittion to the leaf extract. Adebolu and Oladimeji (2005) also did similar test and found out the antimicrobial activity of the leaves of *Ocimum sp.* Parekh et al. (2005) worked on efficacy of aqueous and methanol extracts of some medicinal plants for potential antibacterial activity in twelve medicinal plants. Tomita et al. (1997) reported bactericidal activities of fermented hot water extract from *S. rebaudiana*, towards enterhemorrhagic *E. coli* O157:47 and other food borne pathogenic bacteria. Methanolic extract was found to be the best solvent to result in good antimicrobial activity.

This may be possible that the secondary metabolite "stevioside" is responsible for the antimicrobial activity (Nakamura and Tamura, 1985). It may also be concluded that the secondary metabolite is most soluble and acts as antimicrobial substance when it is in methanolic solvent system. On dilution of the plant leaf extract (for determining of the minimum inhibitory concentration), better susceptibility and zone of inhibition was observed in many cases. This may be due to the pure extract which was more viscous and was unable to permeate and diffuse properly in the medium, but after dilution it could easily diffuse in to the medium (Parekh et al., 2005). The more antimicrobial property of the *in vitro* regenerated plantlets may be due to more secondary metabolite and thus these plantlets can be a source of elite plantlets. Hence this plant can be further subjected to isolation of the therapeutic antimicrobials and further pharmacological evaluation.

REFERENCES

Adebolu TT, Oladimeji SA (2005).Antimicrobial activity of leaf extracts of O*cimum gratissimum* on selected diarrhoea causing bacteria in southwestern Nigeria.. Afr. J. Biotechnol. 4 (7): 682-684.

Akita M, Shigeoka T (1994). Mass propagation of shoots of *Stevia rebaudiana* using a large scale bioreactor. Plant Cell Rep. 13(3-4): 180-183.

Bespalhok FJC, Hattori K (1997). Embryogenic callus formation and histological studies from *Stevia rebaudiana* (Bert.) Bertoni floret explants. Braz. J. Plant Physiol. 9(3): 185-188.

Bespalhok JC, Hashimoto JM, Vieira LGE (1993) Induction of somatic embryogenesis from leaf explants of *Stevia rebaudiana*. Braz. J. Plant Physiol. 5(1): 51-53.

Brandle JE, Starratt AN, Gijzen M (1998) . *Stevia rebaudiana*: its agricultural, biological and chemical properties. Can. J. Plant Sci. 78: 527-536.

Brantner A, Grein E (1994) Antibacterial activity of plant extracts used externally in traditional medicine. J. Ethnopharmacol. 44: 35–40.

Brantner A, Males Z, Pepeljnjak S, Antolic A(1996). Antimicrobial activity of *Paliurus spina-christi* mill. J. Ethnopharmacol. 52: 119–122.

Chalapathi, MV, Thimmegowda S (1997). Natural non-calorie sweetener Stevia (S*tevia rebaudiana* Bertoni), a future crop of India. Crop Res. 14(2): 347-350.

Debnath M, Malik CP, Bisen PS (2006). Micropropagation: A Tool for the Production of High Quality Plant-based Medicines . Curr. Pharm. Biotechnol. 7(1): 33-49.

Erdogrul OT (2002). Antibacterial activities of some plant extracts used in folk medicine. Pharmacol. Biol. 40: 269–273.

Faizi S, Rasool N, Rashid M, Ali Khan RA, Ahmed S, Khan SA, Aqeel Ahmad A, Bibi N, Ahmed SA (2003). Evaluation of the antimicrobial property of *Polyalthia longifolia* var. pendula: isolation of a lactone as the active antibacterial agent from the ethanol extract of the stem. Phytother. Res. 17(10): 1177 - 1181.

Ferreira CM, Handro W (1988). Production, maintenance and plant regeneration from cell suspension cultures of *Stevia rebaudiana* (Bert.) Bertoni. Plant Cell Rep. 7: 123-126.

Flachsland E, Mroginski L, Davina J (1996). Regeneration of plants from anthers of *Stevia rebaudiana* bertoni (Compositae) cultivated *in vitro*. Biocell. 20(1): 87-90.

Megeji NW, Kumar JK, Singh V, Kaul VK, Ahuja, PS (2005) . Introducing *Stevia rebaudiana*. A natural Zero-Calorie sweeteners, Curr. cell sci. 88(5): 801-804.

Murashige T, Skoog F (1962) . A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum 15: 473-497.

Nakamura S, Tamura Y (1985). Variation in the main glycosides of Stevia (*Stevia rebaudiana* Bertoni). Jpn J. Trop. Agric. 29: 109-116.

Nepovim A, Vanek T. (1998) . *In vitro* propagation of *Stevia rebaudiana* plants using multiple shoot culture. Planta Medica 64(8): 775-776.

Ouyang XZ (1996). Plasmalemma invaginations in cultured callus of S*tevia rebaudiana*, ultrastructure and ultracytochemical localization of acid phosphatase. Acta Botanica *Sinica* 38(8): 589-593.

Parekh J, Jadeja D, Chanda S (2005). Efficacy of aqueous and methanol extracts of some medicinal Plants for potential antibacterial activity. Turk. J. Biol. 29: 203-210.

Patil V, Ashwini KS, Reddy PC, Purushotham MG, Prasad TG. , Udaykumar M. (1996) . *In vitro* multiplication of S*tevia rebaudiana.* Curr. Sci. 70(11): 960.

Perez C, Paul M., Bazerque P (1990). An antibiotic assay by the agar-well diffusion method. Acta Biol. Med. Exp. 15: 113-115.

Sah N, Khan M, Vohora SB(1991) . Antimicrobial activity of some medicinal plants extracts on *Escherichia coli*, *Salmonella paratyphi* and *Shigella dysenteriae* Fitoterapia 62 (3): 221-228.

Sakaguchi M, Kan T (1982). Japanese researches on *Stevia rebaudiana* (Bert.) Bertoni and stevioside. Ci Cult. 34: 235-248.

Sikach VO (1998). Effect of nutrient media on physiological peculiarities of stevia rebaudiana Bertoni plants cultivated *in vitro*. Fiziologiya Bikhimiya Kul'turnykh Rastenii 30(4): 294-299.

Sivaram L, Mukundan U (2003). *In vitro* culture studies on *Stevia rebaudiana*. In Vitro Cell. Dev. Biol. 39 (5):520-523.

Tamura Y, Nakamura S, Fukui H, Tabata, M(1984). Clonal propagation of *Stevia rebaudiana* Bertoni by stem-tip culture. Plant Cell Rep. 3: 183-185.

Tomita T, Sato N, Arai T, Shiraishi H, Sato M, Takeuchi M, Kamio Y (1997). Bactericidal activity of a fermented hot-water extract from S*tevia rebaudiana* Bertoni and other food-borne pathogenic bacteria. Microbiol. Immunol. 41(12): 1005-1009.

Xie S, Ouyang XZ, Hong WL. Chen MC, Wang DY, Redai Y, Xuebao Z(1998).The growth and differentiation of callus cultures of *Stevia rebaudiana* in relation to the stevioside accumulation. J. Trop. Subtrop. Bot, 6(1): 8-14.